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Original Article

Effects of curcumin and demethoxycurcumin on amyloid-β precursor and tau proteins through the internal ribosome entry sites: A potential therapeutic for Alzheimer's disease

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Abstract

Objective: This study aims to determine the effects of curcumin and demethoxycurcumin on the internal ribosome entry site of the amyloid-β precursor protein (APP) and tau protein through a bi-cistronic reporter assay for screening of anti-Alzheimer's disease agents.
Materials and Methods: A bi-cistronic assay was performed wherein the expression of the first cistron, a β-galactosidase gene under the control of a cytomegalovirus promoter, represents the canonical cap-dependent mechanism of translation initiation; while the second cistron involves the utilization of the APP or the tau IRES elements to drive the expression of secreted alkaline phosphatase (SEAP) under a cap-independent mechanism. Bioactive natural products reported to have therapeutic potential for AD such as curcumin and demethoxycurcumin were screened in an murine neuroblastoma (N2A) cell model. Western blot analyses for the expression of APP C-terminal protein, human tau-1, and phosphorylated tau at Serine 262 (p^{262}) and Serine 396 (pS^{396}) were done after treatment of N2A cells with the test compounds.
Results: The bi-cistronic reporter assay revealed that curcumin was more effective than demethoxycurcumin, a structural analog of curcumin, in inhibiting both APP and tau IRES-dependent translation initiation. This result was further confirmed by Western blot analysis for the expression of APP C-terminal protein, human tau-1, pS^{262} and pS^{396} suggesting that curcumin may play a role in AD pathology alleviation through the inhibition of the APP and tau IRES-mediated translation mechanism. On the other hand, demethoxycurcumin was observed to inhibit the phosphorylation of both tau pS^{262} and pS^{396}.
Conclusion: A novel assay system using the bi-cistronic reporter constructs for the identification of compounds with activity against the translation directed by APP and tau IRES was developed. The results provide novel suggestive insights for the potential use of the mentioned compounds as prophylactic and therapeutic anti-AD agents.

Keywords: Alzheimer's disease; amyloid-beta; curcumin; demethoxycurcumin; internal ribosome entry sites; tau protein

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Introduction

Demographic aging is a global process that denotes the successes of improved healthcare over the past years. Today, human life expectancy is significantly longer, resulting in a much greater proportion of elderly people. Nevertheless, aging imposes some challenges. It has been well documented that in parallel to aging there is also an increase in cases of Alzheimer's disease (AD) and other dementias in the senile but modern society [1]. Cardinal features of AD pathology include synaptic and neuronal loss, astrocytosis, intraneuronal inclusions of the hyperphosphorylated tau protein in neurofibrillary tangles, and the extracellular deposition of amyloid-β protein in senile plaques. Scientists around the globe have been searching for decades to treat and put an end to the pathological symptoms of AD that deprives our elderly society their intellect, but scientists have met limited success [2]. In spite of the Herculean efforts, only four FDA-approved drugs are currently commercially available in the USA to treat AD pathology [3]. Recent studies demonstrated that the amyloid precursor protein (APP) mRNA may be translated through an internal ribosome entry site (IRES) wherein APP mRNA was found to be one of several mRNAs that may remain associated with polyribosomes during mitosis, when cap-dependent translation initiation is greatly diminished [4]. However, it has also been reported that the 5′ leader in the human tau mRNA contains an IRES and that IRES-dependent translation plays a significant role in the generation of tau protein [5]. Thus, the inhibition of APP and tau expression provides a novel strategy in the continuous battle against neurodegenerative AD. The use of IRES elements to promote internal initiation of translation of RNA has made possible the expression of two or more proteins from polycistronic units in eukaryotic cells. A number of reviews have presented the efficiency of using the bi-cistronic vectors wherein the first gene is translated in a cap-dependent manner while the translation of the second gene is driven by the IRES-dependent mechanism [6,7]. We have developed some recombinant bi-cistronic baculovirus constructs to express pharmaceutically and biotechnologically important recombinant proteins such as interferon-γ (IFN-γ) [8] and Chikungunya virus (ChiKV) 26S [9] and for the screening of drugs or compounds for translation initiation regulation [10].

Plants are the most precise laboratories ever created because they can synthesize metabolites with the most absolute configuration. Many of our available medicines in the market are of plant origin [11]. The drugs available for AD mainly target cholinergic functions associated with the said disease, leaving a vast majority of other potential AD targets nearly unexploited by treatment. Therefore, there is an urgent need for developing drugs based on multiple pathomechanisms of AD. Thus, anti-AD drug design aiming to inhibit the IRES-dependent mechanism of translation initiation was put forward to open new avenues for therapeutic modalities with low cellular toxicity [12,13]. The enormous diversity of functions in natural compounds extracted and isolated from either plant or marine sources may provide a new generation of drugs for AD therapy and management [14,15]. Plant-derived drugs are popular because the public believes that herbs are naturally safer than synthetic drugs. These beliefs may account for the sudden increase in herbal use during recent decades [16].

Curcumin and demethoxycurcumin were selected in this study from a long list of compounds of plant origin to be tested for their ability to inhibit the cap-independent translation mechanism based on the inhibition of APP and tau IRES activities. Curcumin is a bright yellow powder that is also known as deferuloylmethane, natural yellow 3, and (1*E*,6*E*)-1,7-bis(4-hydroxy-3-methoxyphenyl) hepta-1,6-diene-3,5-dione. This substance is isolated from the plant *Curcuma longa*, or turmeric, commonly used for culinary purposes. This substance possesses several functional groups wherein the planar aromatic ring systems are attached to α,β-unsaturated carbonyl groups. The diketones generate stable enols that could be easily deprotonated to form enolates. The α,β-unsaturated group can undergo nucleophilic addition and the Michael reaction. Curcumin has been shown to possess a wide range of pharmacological activities including anti-inflammatory [17,18], antioxidant [19], anticancer [20], wound healing [21], and antimicrobial effects. Demethoxycurcumin, a structural analog of curcumin having one fewer methoxy group, was also isolated from *C longa*. It is an orange powder with a formula weight of 338.35 g/mol. Its IUPAC name is (1*E*,6*E*)-1-(4-hydroxy-3-methoxyphenyl)-7-(4-hydroxyphenyl)hepta-1,6-diene-3,5-dione, and it is also referred to as desmethoxycurcumin, monodemethoxycurcumin, and para-hydroxycurcumin. Demethoxycurcumin shares biological activities with curcumin. Along with curcumin and bisdemethoxycurcumin, the compound demethoxycurcumin has also shown its antioxidant ability based on the reduction of Mo (VI) to Mo (V). The antioxidant capacity of the curcuminoids tested were found to decrease in the order: curcumin > demethoxycurcumin > bisdemethoxycurcumin [22]. This study utilizes a bi-cistronic construct containing the specified IRES flanked by β-galactosidase and secreted alkaline phosphatase (SEAP) genes; the expression of the β-galactosidase gene is controlled by the cytomegalovirus (CMV) while the SEAP expression is under the control of the IRES element.

Materials and methods

Chemicals and reagents

All chemicals and reagents used in this study are of reagent grade. Materials used for cell culture were obtained from Invitrogen, Carlsbad, CA. Fetal calf serum was purchased from Biological Industries, Taipei, TW. Curcumin (C1386, CAS No. 458-37-7) and Demethoxycurcumin (D7696, CAS No. 22608-11-3) were obtained from Sigma-Aldrich, St. Louis, MO. The restriction enzymes used were from Takara, Shiga, JP. Luria broth medium and Luria agar medium were from Lab M Ltd., Lancashire, UK and Alpha Biosciences, Baltimore, MD, respectively. Agarose and bis-acrylamide/acrylamide (29:1, w/v) solution were from AMRESCO,

Solon, OH. Data were obtained from experiments in triplicate and each experiment was repeated three times.

Culturing of cells, plasmid construct, retransformation, and transfection into N2A cells

The cells, murine neuroblastoma (N2A), were cultured in a T-75 flask with 10 mL of culture medium supplemented with 10% fetal calf serum. The cells were allowed to settle to the bottom of the flask during incubation at 37°C in a humidified incubator. After 5 hours, the medium was replenished. The cells were allowed to proliferate to confluency at 37°C in a humidified CO_2 incubator. Cells were passaged every 2–3 days to be used for successive assays. Previously prepared plasmid DNAs were used in this study. The plasmid pUC57 (NCBI No. NM016835) containing the tau IRES gene was synthesized by PROTECH Technology Company, Taiwan. The 240 bp tau IRES was cut from pUC57 by enzymatic digestion with *Not*I and cloned into the *Not*I treated plasmid-pGS-EMCV to replace the EMCV IRES fragment. A similar procedure was done for the insertion of the APP IRES gene into the other pUC57. The APP and the tau IRES contained in pUC57 (NCBI No. NM00084) was amplified by PCR using two pairs of primers: (1Forward: ATTGCGGCCGCAGTTTCCTCGGCAGCGGTAGGCGAGA GCACGCGGAGGAGCGTGCGC; 2Reverse: TCTGCCCGC GCCGCCACCGCCGCCGTCTCCCGGGGCCCCCGCGCA CGCTCCTCCGCGT; 3Forward: TGGCGGCGCGGGCAGA GCAAGGACGCGGCGGATCCCADTCGCACAGCAGCGC ACTC; 4Reverse: TATGCGGCCGCCGCGACCCTGCGCGG GGCACCGAGTGCGCTGCTGTGCGA). The restriction sites in *Not*I are underlined. The plasmids were generated as pGS-APP and pGS-Tau, respectively. In each plasmid, the IRES element from the genes of either APP or tau was flanked by the reporter genes β-galactosidase and SEAP, respectively.

Before transfection, the mammalian cells ($\sim 7-8 \times 10^4$ cells/mL) were seeded on a 24-well plate in serum-free MEM for N2A cells. The cells were allowed to grow and to proliferate up to 90% confluency and the medium was replenished. The plasmids were transfected via liposomes using either TurboFect (Fermentas Life Science, Burlington, ON) or Lipofectamine (Invitrogen) following manufacturers' protocols. Briefly, the plasmid DNA and the liposome (1:2) were mixed in a centrifuge tube. The mixture was allowed to form a complex for 5 minutes at room temperature. The DNA–liposome complex was added to the cells dropwise with gentle mixing by rocking the plate back and forth. The total volume of the medium and DNA–liposome complex was 400 μL. The mixture was then centrifuged at 600× *g* for 10 minutes to allow the DNA–liposome complex to settle. The cells were incubated at 37°C, 95% air/5% CO_2. After 18 hours of incubation, the cells were treated with various concentrations of each compound used in this study.

MTT cytotoxicity assay

The cytotoxic effect of each compound on N2A cells tested in this study was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. This experiment was carried out in parallel to transfection studies. N2A cells at a density of 10^4 cells per well, were treated with desired concentrations of the selected compounds for anti-AD and anti-EV71 screening for 24 hours. Light was avoided throughout the course of the experiment. About 30 μL of MTT solution was added to the 100 μL mammalian cell culture in a 96-well plate. The MTT-treated cells were incubated at 37°C in a humidified CO_2 incubator for 1 hour allowing the development of purple formazan crystals. Afterward, 100 μL of dimethylsulfoxide (DMSO) was added to dissolve the formed formazan crystals. Absorbance was read by a microtiter UV spectrophotometer at a wavelength of 562 nm. Percent survival was calculated using the formula from the paper of Raval et al [23]:

$$\%\ \text{Survival} = \frac{\text{Absorbance}_{\text{TREATED}} - \text{Absorbance}_{\text{CONTROL}}}{\text{Absorbance}_{\text{CONTROL}} - \text{Absorbance}_{\text{BLANK}}}$$

Screening of compounds for IRES activity inhibition

The cells were treated with various concentrations (1 μM, 5 μM, 10 μM, 20 μM, and 40 μM) of curcumin or demethoxycurcumin. After 24 hours treatment, the cell medium was aspirated and kept frozen at −20°C for the SEAP assay. The remaining adherent cells were lysed using 100 μL of Cytobuster protein extraction reagent, centrifugation at 15,800 × *g* for 10 minutes at 4°C followed, and the supernatant was kept at −20°C for the succeeding β-galactosidase assay. Memantine was used as a positive control [24]. The SEAP and β-galactosidase assays were conducted as previously described [10,25].

Western blot analysis

In another independent experiment, the effect of selected compounds on the expression of APP, C-terminal tau proteins phosphorylated at Serine 262 (Tau pS^{262}) and Serine 396 (Tau pS^{396}), and total human tau protein was investigated. Prior to western blot analysis, N2A cells ($7-8 \times 10^4$ cells/well) were seeded onto a 24-well plate and were treated with various concentrations of the selected natural products for 24 hours at 37°C, 95% air/5% CO_2. Then, the culture medium was aspirated and was discarded. The adherent cells were then lysed with 100 μL of Cytobuster protein extraction reagent, centrifugation followed at 15,800 × *g* for 10 minutes at 4°C, and the supernatant was then kept at −80°C until used. Total protein concentration determination for each sample was determined using a bicinchoninic acid assay. Proteins were resolved by denaturing and reducing 8–10% sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) at a constant 80 V for 30 minutes. The voltage was changed to 110 V and proteins were resolved for 2 hours at room temperature. After SDS-PAGE, the proteins were electro-transferred onto a polyvinylidene fluoride (PVDF) membrane (0.45 μm porosity, Immobilon-P, Millipore) at 80 V for 2.5 hours. Immediately after transfer, the membrane was stained

with 1% Ponceau Red in 5% acetic acid to ensure transfer of proteins to the PVDF membrane. Then, the membrane was washed repeatedly with 1× Tris buffered saline to remove the stain. The membrane was then blocked with 5% non-fat milk in Tris buffered saline-0.1% Tween 20 (TBS-T) buffer overnight at 4°C with shaking at 150 rpm. Washing was done three times with TBS-T buffer for 30 minutes (10 minutes/wash, 150 rpm). The individual blocked membrane was then incubated with the respective primary polyclonal antibody at the desired dilution in TBS namely rabbit anti-APP-C-terminal (1:1,000, Cat. No. A8717, Sigma), rabbit anti-TaupS^{262} (1:2,500, Sigma), rabbit anti-TaupS^{396} (1:2,500, Sigma), and rabbit anti-human tau (1:1,500, Sigma). Incubation with primary antibody was performed overnight at 4°C with shaking at 150 rpm. The unbound primary antibodies were removed by washing thrice with TBS-T at room temperature, 150 rpm (10 minutes/wash). Then, the membrane was individually incubated with the anti-rabbit secondary antibody labeled with horseradish peroxidase (1:3,000; Cat. No. 7074; Cell Signaling Technology, Danvers, MA) at 4°C with shaking at 150 rpm for 4 hours. Three-times washing at 150 rpm, 10 minutes per wash, immediately followed to remove the unbound antibodies. Beta-actin (1:10,000, Sigma) was used as an internal control. Proteins were detected using an enhanced chemiluminescence kit (VistaGlow Chemiluminescent Substrate, Cat. No. VG01-500; Visual Protein Biotechnology Corporation, Taipei, TW) following the supplier's protocols. Bands were visualized by Fuji camera along with Fuji Image LAS-3000 software and bands were quantified using the ImageJ Analysis software and were presented as relative area corresponding to the target protein expression. Data presented are results from three independent experiments.

Results and discussion

MTT cytotoxicity assay and screening rationale

The MTT assay is a generally accepted method to determine cell viability. MTT, a yellow solution, is reduced by the mitochondrial dehydrogenases in living cells to a blue-magenta colored formazan precipitate. The absorption of dissolved formazan in the visible region correlates with the number of intact alive cells. Certain compounds may cause damage to cells when added to the culture medium, thereby affecting the viability of the cells [26]. Cytotoxicity evaluation is of paramount importance prior to screening of compounds for anti-AD so as to improve the selection process of potential drugs/plant extracts for the treatment of AD. Thus, curcumin and demethoxycurcumin (Fig. 1) were evaluated for their cytotoxic effects on N2A cells. The results presented in this section were obtained from the MTT assay performed in parallel with transfection studies and western blot analysis. It can be observed in Fig. 2 that the compounds tested against murine N2A cells are relatively nontoxic at the concentrations used. It is also worth noting that demethoxycurcumin exhibits a dose-dependent increase in the N2A cells' viability indicating that it (Fig. 2B) is not toxic within the concentration

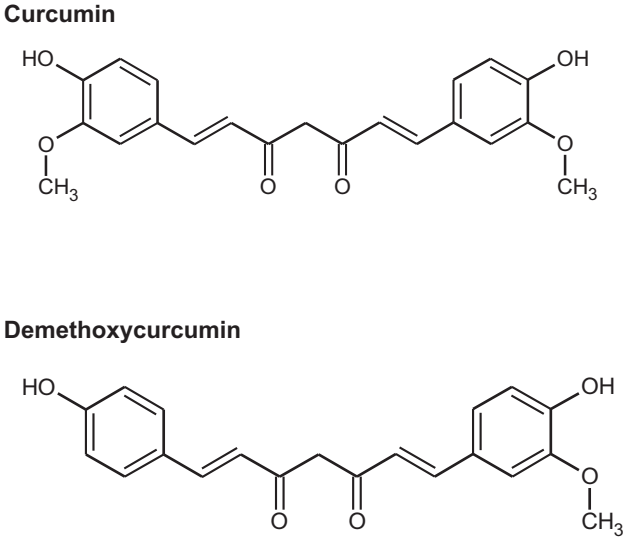

Fig. 1. Structures of curcumin and demethoxycurcumin.

tested. Demethoxycurcumin even promoted the proliferation of N2A cells 24 hours posttreatment as manifested by a 6% increase in the viability as compared to the untreated cells. However, curcumin showed a marginal toxicity resulting to

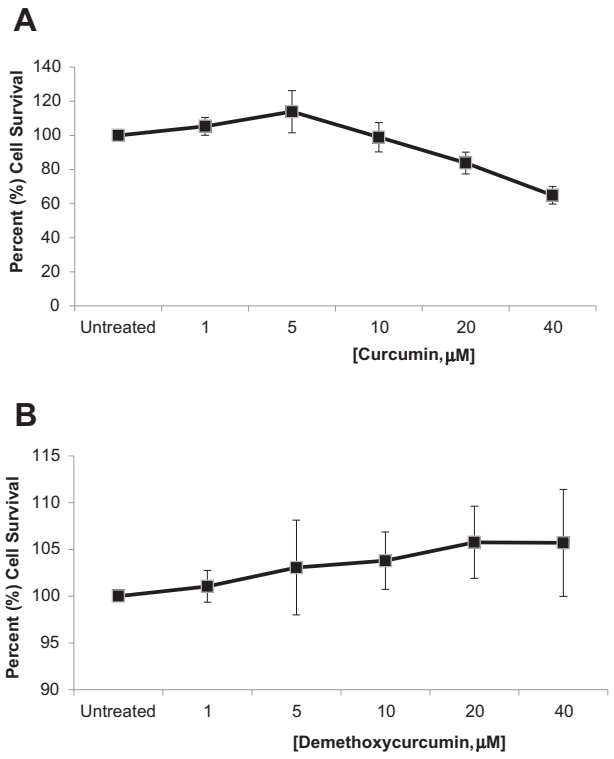

Fig. 2. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay for curcumin and demethoxycurcumin. The neuroblastoma (N2A) cells (~10^4 cells/well) were seeded on a 96-well plate and were subsequently treated with different concentrations of (A) curcumin and (B) demethoxycurcumin. At 24 h after treatment with these compounds, 30 μL of thiazolyl blue tetrazolium bromide (5 mg/mL) was added. The purple formazan crystals formed were dissolved in dimethylsulfoxide (DMSO), and the absorbance at 562 nm was measured on a spectrophotometer.

about 60% survival in N2A cells at a concentration of 40 μM, (Fig. 2A). It was also noted that curcumin did not cause any toxic effect at concentrations <10 μM.

The expression of APP and tau proteins has been demonstrated in a number of reported studies to be mediated by the IRES, an atypical translational initiation mechanism, aside from the conventional cap-dependent translation initiation [4,5]. It has been assumed that it could be possible to identify compounds that may be able to distinguish between cap-dependent translation and cap-independent IRES-mediated translation. In the facilitation of compound screening, bi-cistronic reporter constructs were developed containing a β-galactosidase gene and a SEAP reporter gene. In each bi-cistronic expression vector, the β-galactosidase and SEAP reporter genes are under the transcriptional control of the human cytomegalovirus major intermediate early promoter/enhancer sequence (CMV promoter). Directly after transcription, the β-galactosidase gene is translated by a cap-dependent mechanism, while SEAP expression is directed by either APP (Fig. 3A) or tau IRES (Fig. 3B). This model can possibly recognize compounds that can stall SEAP expression representing the cap-independent translation without affecting β-galactosidase activity that represents cap-dependent translation initiation in transfected cells.

Curcumin but not demethoxycurcumin affects APP-IRES-mediated translation initiation in murine neuroblastoma cells

Established through various studies, turmeric, a popular spice, is useful in the therapeutic management of AD [27]. Here, curcumin and its structural analogue, demethoxycurcumin were tested for their ability to inhibit the APP-IRES-directed translation initiation in a transfected mouse neuroblastoma cell line. In Fig. 4, curcumin was able to cause a minimal effect on the SEAP activity at concentrations of 1–20 μM (up to 20% inhibition of SEAP activity). At these concentrations, the β-galactosidase activity was not significantly affected by the treatment of N2A cells with curcumin. This implicates that curcumin was able to inhibit the APP-IRES activity without affecting the cap-dependent mechanism. Treating the transfected cells with 40 μM curcumin resulted in a more pronounced inhibition of SEAP, but the β-galactosidase activity was reduced to almost 60% and if this result is correlated with the result of the MTT assay, it can be deduced that curcumin is relatively nontoxic at a concentration <10 μM. In contrast, demethoxycurcumin did not inhibit the APP-IRES-dependent translation initiation in transfected mouse neuroblastoma cells as shown in Fig. 5. Within the concentration range used (1–40 μM) demethoxycurcumin promoted the activity of SEAP; therefore, there is an observed promotion of the APP-IRES-mediated translation initiation. The β-galactosidase assay showed that increasing the amount of demethoxycurcumin from 1 μM to 40 μM in the culture medium favors the expression of β-galactosidase protein within the cell, thereby increasing the occurrence of cap-dependent translation initiation within the N2A cells. Similarly, the result of the MTT assay for demethoxycurcumin is consistent with the β-galactosidase assay.

Curcumin is more active in attenuating APP expression than demethoxycurcumin

Based on the results presented in the previous section, curcumin was able to cause an effect on the APP-IRES-mediated translation initiation while demethoxycurcumin seemed to favor the activity of APP-IRES. It was hypothesized that these compounds would have a similar pattern of effect on the expression of APP in the mouse neuroblastoma cells. The N2A cells were treated with 1 μM, 5 μM, 10 μM, 20 μM, and 40 μM of either curcumin or demethoxycurcumin for 24 hours. Subsequently, the cell lysates were subjected to western blot analysis to investigate the effect of the said compound on the expression of particularly the C-terminal of human APP_{695} (676–695). APPs are members of a large family of transmembrane glycoproteins widely distributed in many tissues. APP has three major isoforms: APP_{695}, APP_{751}, and APP_{770}. Both APP_{751} and APP_{770} contain a 56 amino acid domain. APP_{695} is preferentially expressed in the central nervous system while the other two are more abundant in the peripheral nervous system [28]. APP is thought to be internalized and degraded by an endosomal–lysosomal pathway to yield amyloidogenic peptides that are cytotoxic and cause neuronal damage and degeneration. As quantified using the ImageJ software, the results of the western blot analysis were in agreement with the data obtained from the SEAP reporter

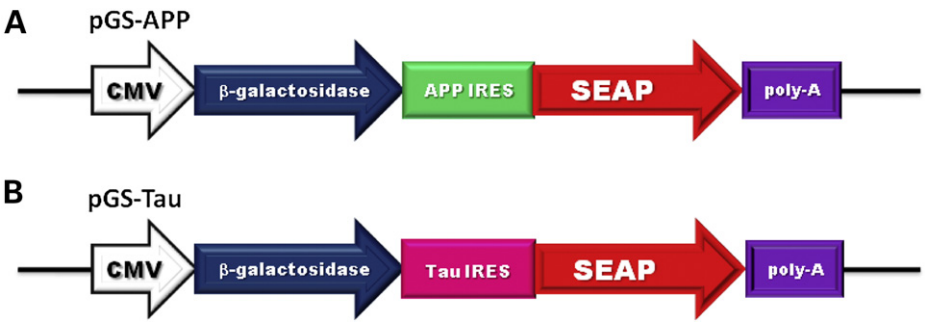

Fig. 3. DNA plasmid constructs. These constructs contain the elements from (A) amyloid precursor protein (APP) and (B) Tau internal ribosome entry site (IRES). The plasmids use the cytomegalovirus (CMV) promoter to drive the bi-cistronic mRNA transcription in transfected mammalian cells.

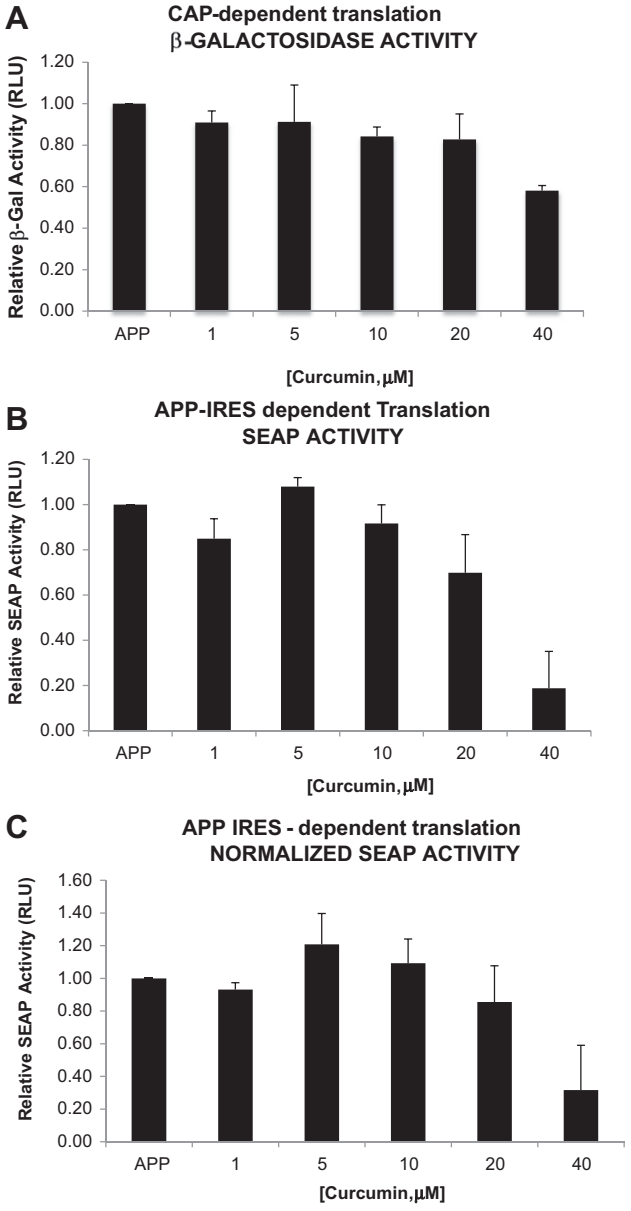

Fig. 4. Effects of curcumin on cap-dependent and amyloid precursor protein-internal ribosome entry site (APP-IRES)-mediated translation initiation in neuroblastoma cells. About $7-8 \times 10^4$ N2A cells were seeded on a 24-well plate. A DNA plasmid containing IRES elements of the 5′UTR amyloid precursor protein (pGS-APP) was transfected using Turbofect reagent with centrifugation at $600 \times g$ at 26°C for 10 min. Cells were allowed to proliferate and were treated with various concentrations of curcumin (1 μM, 5 μM, 10 μM, 20 μM, and 40 μM). After 24 h, the cell medium was aspirated and subjected to a secreted alkaline phosphatase (SEAP) activity assay while the adherent cells were lysed by Cytobuster and lysates were subjected to a β-galactosidase assay.

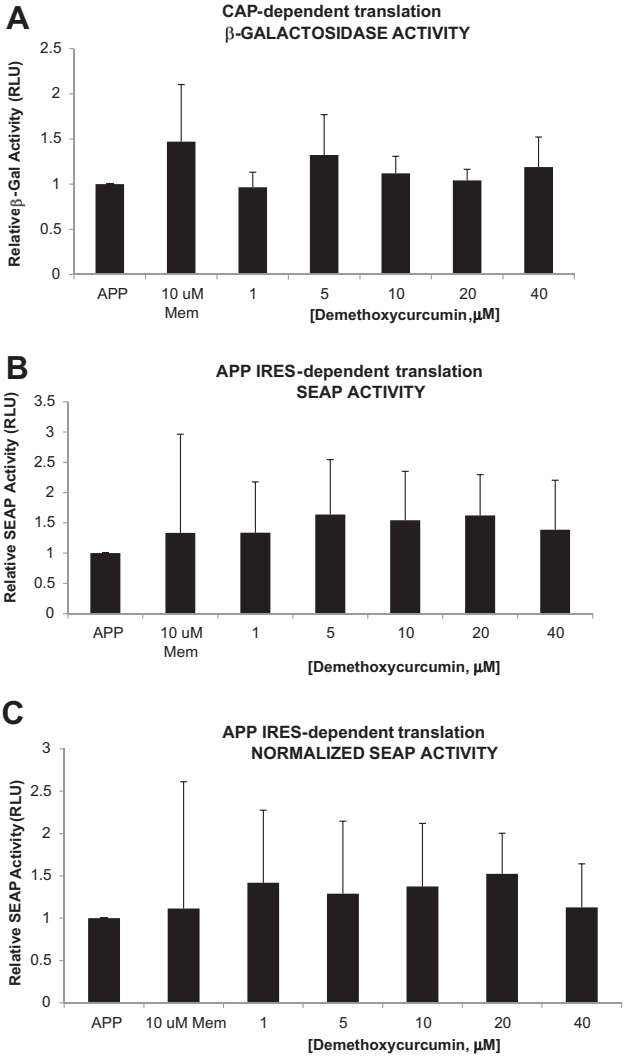

Fig. 5. Effects of demethoxycurcumin on the cap-dependent and amyloid precursor protein-internal ribosome entry site (APP-IRES)-mediated translation initiation in neuroblastoma cells. About $7-8 \times 10^4$ N2A cells were seeded on a 24-well plate. A DNA plasmid containing IRES elements of the 5′UTR amyloid precursor protein (pGS-APP) was transfected using Turbofect reagent with centrifugation at $600\times g$ at 26°C for 10 min. Cells were allowed to multiply and were treated with various concentrations of demethoxycurcumin (1 μM, 5 μM, 10 μM, 20 μM, and 40 μM) for 24 h. Then the cell medium was aspirated and subjected to a secreted alkaline phosphatase (SEAP) activity assay while the adherent cells were lysed by Cytobuster and lysates were subjected to a β-galactosidase assay.

assay, where curcumin was able to cause an inhibitory effect on the APP-IRES-mediated translation initiation (Fig. 6A). It can be seen that upon treating N2A cells with increasing amounts of curcumin, the level of APP expression is decreased significantly, whereas treatment of N2A cells with demethoxycurcumin at increasing concentrations resulted in a promotion of APP expression (Fig. 6B). These data suggest that curcumin and not demethoxycurcumin may play a role in the treatment of AD through the modulation of APP-IRES activity. This is because demethoxycurcumin, at doses tested, was able to promote APP-IRES activity as seen on the increased expression of APP in the western blot analysis. Zhang and colleagues investigated the effect of curcumin on the metabolism of amyloid-β and APP in various cell lines including rat neuroblastoma cells overexpressing human APP_{751}. Curcumin significantly increased the levels of matured APP at low concentrations (5 μM and 10 μM), and there was an observed decrease in the level of matured APP at 15 μM and 20 μM. As a result, curcumin treatment markedly increased the ratio of matured APP and immature APP at

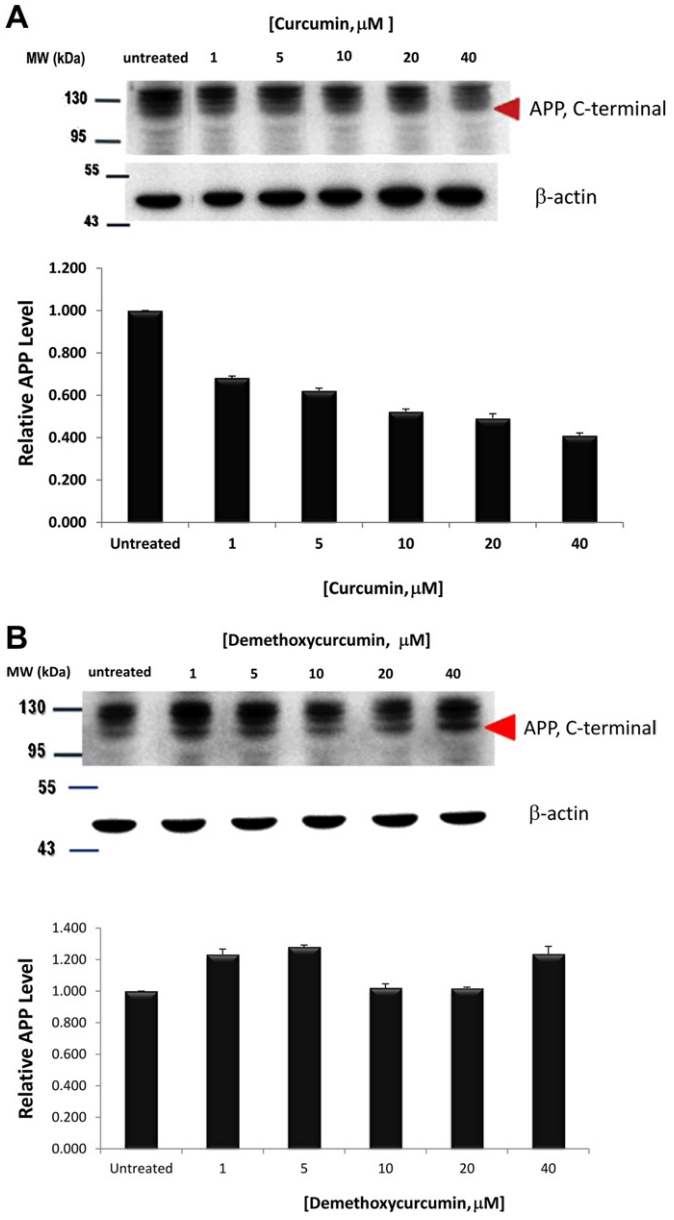

Fig. 6. Effects of (A) curcumin and (B) demethoxycurcumin on APP C-terminal protein expression. N2A cells ($\sim 7-8 \times 10^4$) were seeded on a 24-well plate, and these were treated with various concentrations of either curcumin or demethoxycurcumin (1 μM, 5 μM, 10 μM, 20 μM, and 40 μM). The cell lysates were subjected to western blot analysis. Total protein concentration was measured using BCA Total Protein Kit (Pierce). Proteins (40 μg) were resolved by 8% sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by electrotransferring onto a polyvinylidene fluoride (PVDF) membrane. Detection of APP C-terminal protein was accomplished by the use of rabbit polyclonal anti-APP, C-terminal primary antibody (1:2500), and incubation with secondary anti-rabbit HRP conjugated antibody (1:3000). Bands were visualized using VistaGlow Chemiluminescent Substrate. Bands were quantified using ImageJ software and were presented as relative area corresponding to APP expression. Data presented are results from three independent experiments.

lower concentrations, and again the ratio was significantly decreased at higher concentrations of curcumin [29].

Curcumin and demethoxycurcumin are structural analogues and, because demethoxycurcumin has one methoxy group fewer than curcumin, it can be deduced that the methoxy group is important in the curcumin molecule in terms of its ability to act on the IRES elements of APP. The methoxy group also gives curcumin a more hydrophobic character, making it capable of penetrating the plasma membrane more easily compared to demethoxycurcumin.

On a different note, demethoxycurcumin, as mentioned above, increased the expression of APP in untransfected neuroblastoma cells consistent with the SEAP activity data from the transient transfection of pGS-APP in N2A cells. However, the addition of demethoxycurcumin to N2A cells did not substantially affect the β-galactosidase activity, in this manner the cap-dependent translation within the cell was not affected by demethoxycurcumin treatment. In connection with this, the MTT assay result for demethoxycurcumin-treated N2A cells showed an increased viability with increasing concentrations of demethoxycurcumin. A previous *in vitro* study showed that curcuminoids isolated from *Curcuma longa* were able to protect PC12 pheochromocytoma cells and human umbilical vein endothelial cells from amyloid-β_{1-42} insults [30]. Considering the structure of curcuminoids, the protection effect is due to the presence of the 4-hydroxy group on the cinnamate portion of the compound while the 3-hydroxy group is less effective in protecting the cells [31]. Based on the presented results and observations, it could be possible that demethoxycurcumin may play a protective role in murine neuroblastoma cells.

Curcumin and demethoxycurcumin exhibit opposing effects on tau IRES-mediated translation initiation

Studies have shown that curcuminoid compounds are able to attenuate tau phosphorylation and tau pathologies. Curcumin and demethoxycurcumin were tested for their ability to inhibit the tau IRES-mediated translation initiation *in vitro,* using pGS-Tau transfected N2A cells. It is observed in Fig. 7 that curcumin minimally caused an effect on the cap-dependent translation in N2A cells at concentrations less than 10 μM based on the β-galactosidase assay. This observation is consistent with the findings of the MTT assay for curcumin against N2A cells proving that curcumin is not cytotoxic to N2A cells at 10 μM and below. However, there was an observed dose-dependent decrease in the beta-galactosidase activity at concentrations above 10 μM, that is, about 20% at 20 μM and 50% at 40 μM curcumin as compared to the β-galactosidase activity in the untreated cells. Interestingly, curcumin promoted tau-IRES-mediated translation as seen on the measured SEAP activity in the cell medium of 1 μM, 5 μM, and 10 μM curcumin-treated N2A cells. Furthermore, a dose-dependent increase in the SEAP activity was observed at these concentrations. There was significant reduction in the SEAP activity at 20 μM (70% inhibition) and 40 μM (80% inhibition). However, these concentrations may have some detrimental effects when used because curcumin affected the viability as well as the cap-dependent translation in N2A cells. Demethoxycurcumin enhanced the activity of SEAP after addition to N2A cells at 1 μM, 5 μM, 10 μM,

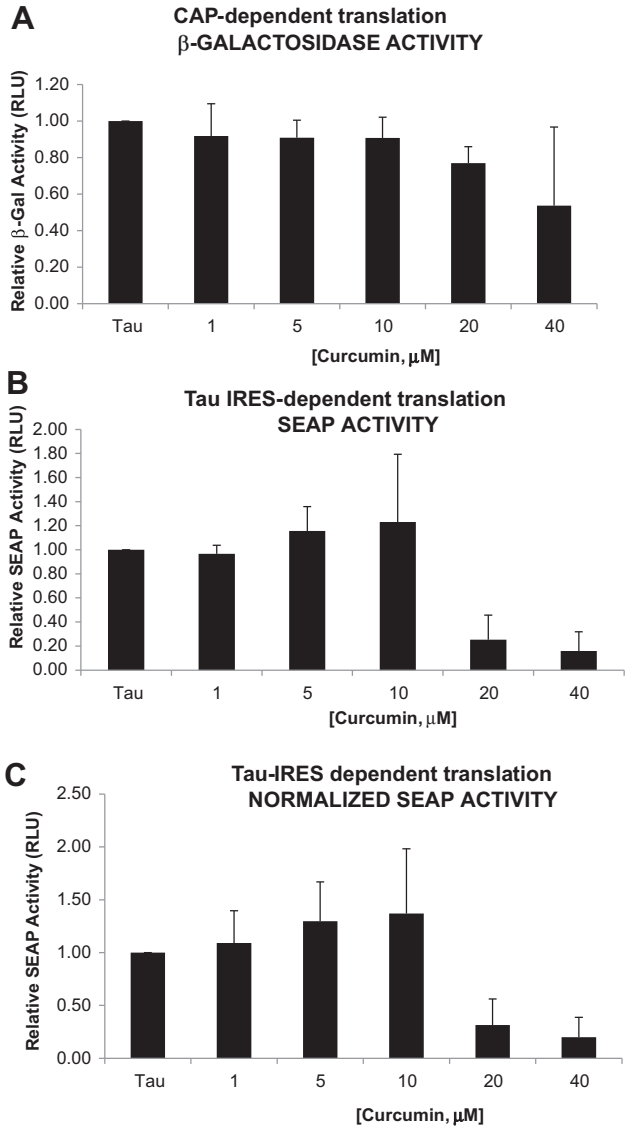

Fig. 7. Effect of curcumin on the cap-dependent and tau internal ribosome entry site (IRES)-mediated translation initiation in neuroblastoma cells. About $7-8 \times 10^4$ N2A cells were seeded on a 24-well plate. A DNA plasmid containing IRES elements of the 5′UTR amyloid precursor protein (pGS-Tau) was transfected using Turbofect reagent with centrifugation at $600 \times g$ at 26°C for 10 min. N2A cells were allowed to proliferate and were treated with various concentrations of curcumin (1 μM, 5 μM, 10 μM, 20 μM, and 40 μM). After 24 hours, the cell medium was aspirated and subjected to a secreted alkaline phosphatase (SEAP) activity assay while the adherent cells were lysed by Cytobuster and lysates were subjected to a β-galactosidase assay.

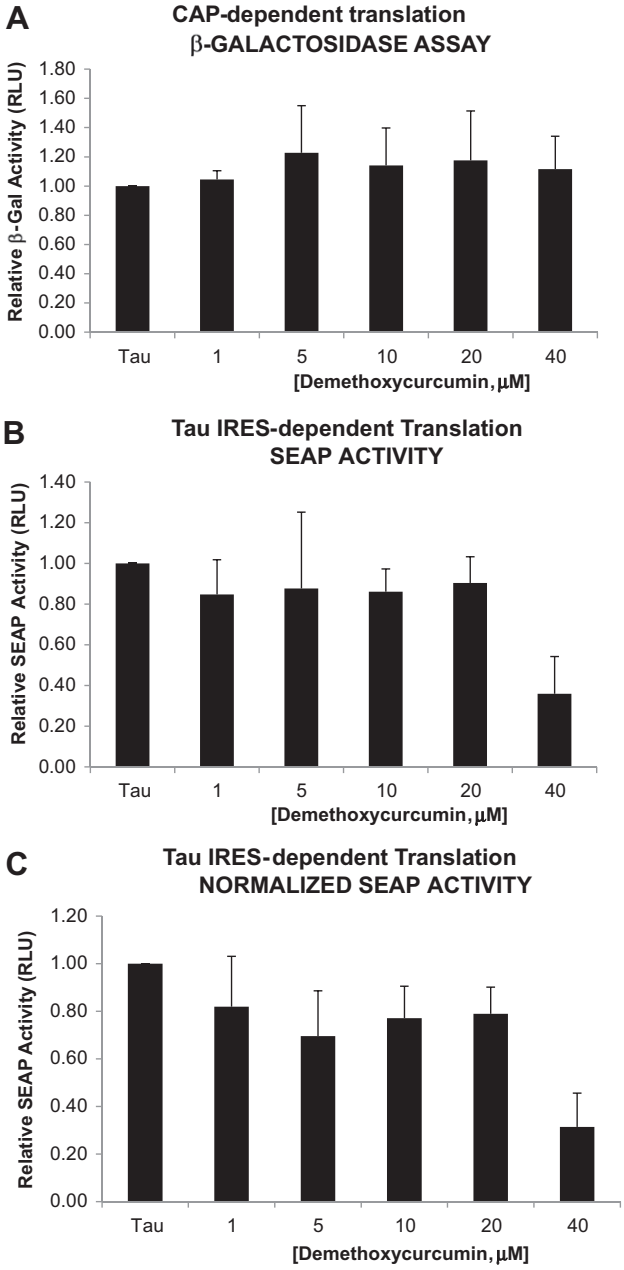

Fig. 8. Effect of demethoxycurcumin on tau-internal ribosome entry site (IRES)-dependent translation in murine neuroblastoma cells. About $7-8 \times 10^4$ N2A cells were seeded on a 24-well plate. A DNA plasmid containing IRES elements of the 5′UTR amyloid precursor protein (pGS-Tau) was transfected using Turbofect reagent with centrifugation at $600 \times g$ at 26°C for 10 min. Cells were allowed to proliferate and were treated with various concentrations of demethoxycurcumin (1 μM, 5 μM, 10 μM, 20 μM, and 40 μM). After 24 h, the cell medium was aspirated and subjected to a secreted alkaline phosphatase (SEAP) activity assay while the cells were lysed by Cytobuster and lysates were subjected to a β-galactosidase assay.

20 μM, and 40 μM (Fig. 8); a dose–activity relationship was not observed from the SEAP assay, while the β-galactosidase assay was in agreement with the MTT assay data for demethoxycurcumin on N2A cells.

Effects of curcumin and demethoxycurcumin on the expression of total tau and phosphorylated tau

The tau protein is a microtubule-associated protein present in brain and other neuronal tissues. It is expressed predominantly in axons of both the central and the peripheral nervous system, but is barely detectable in astrocytes or oligodendrocytes. Total and phosphorylated tau proteins are considered promising candidate biomarkers for AD. Phosphorylation at some sites in the tau protein has been reported to play a dominant role in the reduction of the binding capacity of tau to the microtubule. Of the 79 potential serine and threonine phosphorylation sites, 39 have already been verified [32].

Immunochemical and protein chemical approaches revealed that Serine 262 (pS^{262}) was phosphorylated by CaM kinase II as well as cAMP-kinase and MAP/microtubule affinity regulating kinase [33]. Furthermore, tau protein in AD brain was found to be phosphorylated at Serine 396 (pS^{396}) [34]. In this study, the levels of total tau protein as well as the phosphorylated tau at Serine 262 and Serine 396 were determined after the treatment of mouse neuroblastoma cells with curcumin and demethoxycurcumin.

Based on Fig. 9A, N2A cells were treated with curcumin at different doses such as 1 μM, 5 μM, 10 μM, 20 μM, and 40 μM. After 24 hours treatment, the cells were lysed and the lysates were subjected to western blot analysis. Using ImageJ software, the levels of the specific tau protein were quantified as the area of each peak corresponding to the bands visualized on the PVDF membrane by the enhanced chemiluminescence detection. Beta-actin was used as internal control to normalize the level of the tau expression. It was observed that at low

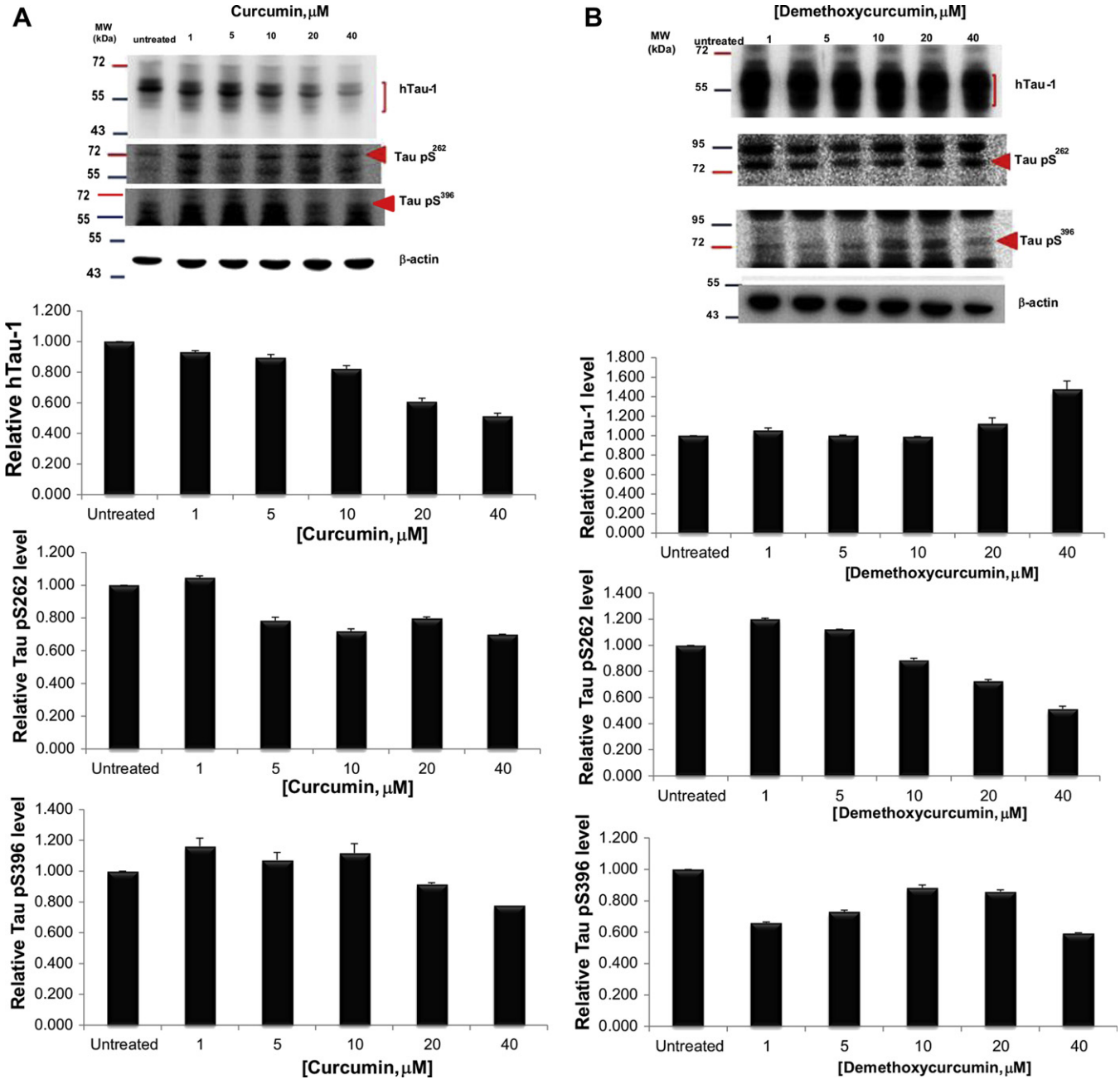

Fig. 9. Effect of (A) curcumin and (B) demethoxycurcumin on total tau (human tau-1) and phosphorylated tau pS^{262} and tau pS^{396} in murine neuroblastoma cells. Cells (~7–8 × 10^4 cells/well) were treated with various concentrations of curcumin or demethoxycurcumin (1 μM, 5 μM, 10 μM, 20 μM, and 40 μM) for 24 h. The cell lysates (40 μg protein) were subjected to 8% sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot analysis using antibodies against human tau-1 (1:1500), Tau pS^{262} (1:2500) and Tau pS^{396} (1:2500) and secondary anti-rabbit HRP (1:3000). Detection and visualization was done using VistaGlow Chemiluminescent Substrate kit. The bands were quantified using ImageJ Software.

doses (1–10 μM) curcumin did not significantly alter the amount of the total tau. However, at concentrations of 20 μM and 40 μM, the amount of the total tau (hTau-1) was dose-dependently decreased by 40% and 50%, respectively. Furthermore, the degree of tau pS^{262} was decreased markedly by up to 30% upon treatment of cells with 5–40 μM curcumin. The amount of tau pS^{396}, however, was increased compared to the untreated cells by approximately 20%. It is noticeable that at 1 μM curcumin both tau pS^{262} and pS^{396} were present, although this had very little effect on the hTau-1 level. It is worth noting that at 20 μM and 40 μM curcumin, the amounts of pS^{262} and pS^{396} were almost equivalent.

By correlating the results of the western blot analysis with those of the MTT assay, it can be deduced that curcumin is relatively safe for N2A cells at concentrations <10 μM. The increased viability of N2A at concentrations less than 10 μM curcumin from the MTT assay may be attributed to the maintenance of the integrity and stability of the microtubules inferred from the result obtained from western blot analysis for total hTau-1. Western blot analysis of cell lysates from the demethoxycurcumin-treated N2A cells revealed that 1–10 μM demethoxycurcumin did not elicit any effect of the hTau-1 expression but higher concentrations of demethoxycurcumin such as 20 μM and 40 μM obviously increased the expression of hTau-1 in N2A cells (Fig. 9B). Interestingly, it can be discerned that demethoxycurcumin exhibited a dose-dependent reduction in the expression of tau pS^{262} at 10 μM, 20 μM, and 40 μM concentrations while 1 μM and 5 μM demethoxycurcumin marginally increased the expression of tau pS^{262}. Analysis of the level of expression showed that demethoxycurcumin was able to diminish the amount of tau pS^{396} at all concentrations tested. It is ascertained that the curcuminoids (curcumin and demethoxycurcumin) may play a potential role in treatment of AD via the modulation of tau protein expression and its phosphorylation. Curcumin can act as a promoter of tau IRES-mediated translation initiation at concentrations less than 10 μM and may act as inhibitor of the tau IRES at higher concentration, i.e., 20 μM, without significantly affecting the cap-dependent translation machineries with the cell. Consistently, curcumin was able to maintain the amount of total tau proteins that may account for the viability of N2A cells at concentrations <10 μM. Although demethoxycurcumin was not able to inhibit the tau IRES-dependent translation initiation in murine N2A cells, the results from the western blot imply that demethoxycurcumin can prevent AD through its action on the stalling of the phosphorylation of tau at Serines 262 and 396, apart from the protective role it gave to the cells.

Conclusion

This study presented the use of a bi-cistronic reporter assay as a method to determine the effects of curcumin and demethoxycurcumin on AD by the use of IRES elements housed in a single expression vector. In this bi-cistronic construct, the IRES element (APP and Tau) is flanked by gene sequences from β-galactosidase and SEAP. The expression of the β-galactosidase protein was controlled by the cytomegalovirus promoter, which corresponded to the cap-dependent translation. The expression of SEAP was under the control of the IRES element, which represented the cap-independent translation. Both APP and Tau IRES were functional in the neuroblastoma cells. Natural compounds used for the study were observed to be of potential use for the treatment of either AD or enteroviral infection. Curcumin was more active than demethoxycurcumin in inhibiting the APP and Tau IRES activity because demethoxycurcumin failed to inhibit APP–IRES but was able to reduce Tau activity only at the highest concentration tested equal to 40 μM. Western blot analysis further proved the usefulness of the compounds tested for AD. Curcumin was able to attenuate the expression of APP, C-terminal protein, tau pS^{262}, and tau pS^{396} while demethoxycurcumin failed to inhibit APP and C-terminal protein. On the contrary, demethoxycurcumin was able to halt the expression of tau pS^{262} and tau pS^{396}. The mechanism to explain the interactions between the IRES elements and the curcuminoids needs to be addressed in the future. The bi-cistronic vector reporter assay provided a novel, simple, and effective platform in screening and establishing the mechanistic action of potential compounds for the treatment and management of AD.

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